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Article

In vitro acaricide activity of extracts from three *Leucaena* spp. genotypes versus *Rhipicephalus microplus*

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Abstract:

The tick *Rhipicephalus microplus* is known to develop resistance against some commercial acaracides, driving a search for natural alternatives. An evaluation was done of the acaricide activity against adult and larval *R. microplus* of ethanol extracts from three *Leucaena* spp. genotypes: *L. leucocephala* (Lam.) de Wit (Native); *L. leucocephala* (Cunningham); and *L. Leucocephala x L. padilla* (KX2). Larval immersion and adult immersion tests were used to evaluate acaricide activity. Secondary metabolite profiles of the three genotypes were generated using analytical chromatographic plates. Against the larvae, the 50% extract concentration exhibited 91.68% mortality for the Cunningham genotype, 82.00% for the KX2 and 54.06% for the Native. The Native genotype extract was most effective against adults with a 50% mortality at a 20% concentration. Flavonoids and terpenes were identified in all three genotypes and are probably responsible for their acaricide activity. The *Leucaena* spp. Cunningham and KX2 extracts were effective against *R. microplus* larvae, but further research is needed to identify the metabolites that provide this acaricide activity, be it individually or synergistically.

Key words: Ectoparasites, Secondary metabolites, Vegetal extracts, Subhumid tropics.

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Introduction

The tick *Rhipicephalus microplus* is among the ectoparasites which cause the greatest losses in livestock. It is also a vector for pathogenic agents such as *Anaplasma marginale*, *Babesia bigemina* and *B. bovis*[(1)]. Worldwide an estimated 80% of cattle are infested with ticks, resulting in annual losses from two to three billion US dollars. Annual losses due to *R. microplus* infestations in cattle in Mexico were recently estimated to be 573.61 million USD[(2)].

The most common way of controlling *R. microplus* is the use of chemical compounds such as pyrethroids (PT), organophosphates (OP), amidines (AM), phenylpyrazolones, tick growth inhibitors and macrocyclic lactones. However, in recent years an increase in ixodicide-multiresistant strains (mainly PT, OP and AM) has been reported in southeastern Mexico[(3)]. There are also now reports of *R. microplus* resistant to ivermectin[(4)], and to fipronil[(5)]. This situation is driving a search for alternative methods for controlling *R. microplus* that also reduce potential damage to the environment and humans[(1)].

Plant extracts are promising alternative approaches to arthropod control. They produce secondary metabolites with different action mechanisms such as inhibition of feeding and chitin synthesis; decreased growth, development, and reproduction; and behavioral alterations; this, without adverse effects to non-target species[6]. Extracts from plants in the families Lamiaceae, Fabaceae, Asteraceae, Piperaceae, Verbenaceae and Poaceae exhibit the greatest efficacy in tick control[7-11]. Secondary metabolites with an acaricide effect versus *R. microplus* have been identified in plant extracts from *Cunila angustifolia, Acacia pennatula, Piscidia piscipula, Leucaena leucocephala, Tagetes minuta, Piper amalago, Lippia graveolens* and *Milinis minutiflora*. These include terpenes, stilbenes, coumarins, acids, alcohols, sulfide compounds, tannins, and aldehydes from essential oils[7,12].

Few studies have been done to date on the effect of *Leucaena* spp. extracts in tick control. For example, one study assessed the effect of a *L. leucocephala* extract on *R. microplus* and found 66.79 % efficacy versus larvae, but none against adults[12]. In contrast, a study of defensive proteins produced by *L. leucocephala* (Lam.) de Wit were found to have an effect against adult *R. microplus* (56.3 % efficacy)[13].

Three *Leucaena* genotypes are currently available in southeast Mexico: *L. leucocephala* (Native); *L. leucocephala* (Cunningham); and *L. leucocephala* x *L. padilla* (KX2). These are widely used as forage shrub plants in tropical silvopastoral systems, and may also contribute to controlling arthropods. The present study objective was to evaluate *in vitro* the acaricide activity of ethanol extracts from these three *Leucaena* genotypes against *R. microplus* adults and larvae.

Materials and methods

Experimental site

The study was done at the Maya Zone Technological Institute (Instituto Tecnológico de la Zona Maya - ITZM), Carretera Chetumal–Escárcega Km. 21.5, Ejido Juan Sarabia, Municipality of Othón P. Blanco, in the state of Quintana Roo, Mexico (18°30”58’ N; 88°29”19’ W). Average maximum temperature in the area is 32.1 °C, and the average minimum is 21.7 °C. Annual rainfall averages 1,180 mm, and average relative humidity (RH) ranges from 76 to 82% (http://smn.conagua.gob.mx).

Rhipicephalus microplus larva production

A total of 300 engorged adult ticks were collected from at least 25 cattle on the Las Flores Ranch located in the town of Xul-ha, Quintana Roo, Mexico. The ticks were placed in glass tubes with cotton lids and transported to the ITZM Biological Control Laboratory. Here they were washed, dried and weighed. Average tick weight was 200 ± 20 mg. One group of engorged ticks was used in the adult immersion test (AIT) and another group was incubated at 27 ± 1.5 °C and 70 to 80% RH for two weeks to allow oviposition[14]. The eggs were transferred to 10 ml glass vials with a cotton lid. Larvae hatched approximately 30 days after collection of engorged females. Larvae from 7 to 14 days of age were used in the larval immersion test.

Vegetal material

Three *Leucaena* spp. genotypes were included in the study: *L. leucocephala* (Lam.) de Wit (native); *L. leucocephala* (Cunningham); and *L. leucocephala* x *L. padilla* (KX2). A total of 25 kg of fresh leaves were collected from each genotype. These were dried at 60 ºC for 72 h, and ground with an electric Wiley mill (Thomas Scientific®) to a 3 mm particle size.

Extract production

At the ITZM laboratory the ground *Leucaena* leaves were submerged in 80% ethanol (absolute ethanol) for 72 h (800 ml methanol and 350 g ground leaves per genotype). Ethanol was used because it is a general polar solvent that extracts compounds of interest (e.g. terpenes and flavonoids) from vegetal material. Each ethanol extract was filtered and evaporated at 45 °C in a rotational evaporator under a vacuum. The resulting crude extracts were transferred to glass flasks and kept at 4 °C until use.

Bioassay with larvae

Efficacy of the three *Leucaena* genotypes was quantified by larval bioassays using the larvae immersion test as modified by Soberanes *et al.*[15]. Six concentrations (50, 40, 30, 20 and 10%) of each *Leucaena* genotype extract were tested. Each concentration was transferred to Petri dishes (60 mm x 15 mm diameter), 300 to 500 larvae placed between two sheets of Whatman No. 1 paper,

and these submerged for 10 min in the extract. Approximately 100 larvae were collected with a brush (No. 4), gently transferred to an envelope of clean filter paper previously marked with identifying information, and this sealed with a metal clip. The control group was exposed to Tween® 20 (2%) and water. Three repetitions were tested per concentration. The envelopes were placed on trays and incubated for 48 h at 27 ± 2 °C and 80 to 90% (RH). After incubation, counts were done of live and dead larvae. Those larvae capable of walking were considered to be alive while those exhibiting an absence of movement, ataxia or movement of appendices were considered dead. Mortality rate was calculated with an established formula[16], as was the efficacy of the different genotypes[17].

% Mortality = dead larvae/total larvae x 100
Average % mortality = (mortality 1 + mortality 2 + mortality 3)/3
% Efficacy = (control group-treated group)/control group x 100

Bioassay with adults

Twelve homogeneous groups of ten engorged ticks (average weight = 200 ± 20 mg) each were formed. The same *Leucaena* spp. genotype extracts were used but only at 20 and 10% concentrations; three repetitions were done per concentration. The treatments were immersed in the corresponding extract and concentration for one minute while the control was immersed in Tween® 20 (2%)[18]. The treated ticks were placed in a 24-well culture plate, one per well, and incubated for fifteen days under the temperature and RH conditions described above. Mortality and survival rates were recorded daily using a stereoscope. At day fifteen, the eggs produced in each group were weighed. One hundred of these eggs were placed in glass vials under the same incubation conditions for 21 d, after which hatch rates were estimated for each treatment and these compared to the control.

Thin-layer chromatography (TLC)

Secondary metabolite profiles for the three genotypes were determined using TLC silica gel 60 F_{264} analytical plates (Merck®) and silica gel 60 70-230 mesh for gravity chromatography (Sigma Aldrich®). Three chromatographic revealers were used: ceric sulfate (universal revealer); oleum (terpene identification); and stannous chloride (flavonoid and terpene detection)[19].

Statistical analyses

Established formulas were used to calculate larval mortality[16], and efficacy[17]. Reproductive efficiency (RE) and the percentage reduction in estimated reproduction (PRER) were calculated with a reported equation[20]. The lethal concentration at 50% (LC_{50}) and its confidence interval at 95% (CI_{95}) were determined with the Probit analysis program. Reproductive efficiency (RE) and PRER were calculated with these formulas:

RE = egg mass weight X % larvae hatched/initial tick weight
PRER = Control group RE – Treated Group RE/Group Control RE X 100

Results

Larval mortality

The 50% Cunningham genotype extract caused 91.7 % mortality of *R. microplus* larvae and had 92.7 % efficacy. The 50% KX2 genotype extract caused 82.0 % mortality and had 90.7 % efficacy. Activity was notably lower in the Native genotype (54.6 % mortality, 75.6 % efficacy). Calculation of the LC_{50} found that the KX2 extract required 15.8 % (11.9 ± 19.0) of the concentration to reach this benchmark, while the Cunningham extract required 22.2% (19.2 ± 25.3) and the Native extract 43.7 % (36.0 ± 19.0).

Adult mortality, reproductive efficiency index and reduction in estimated reproduction

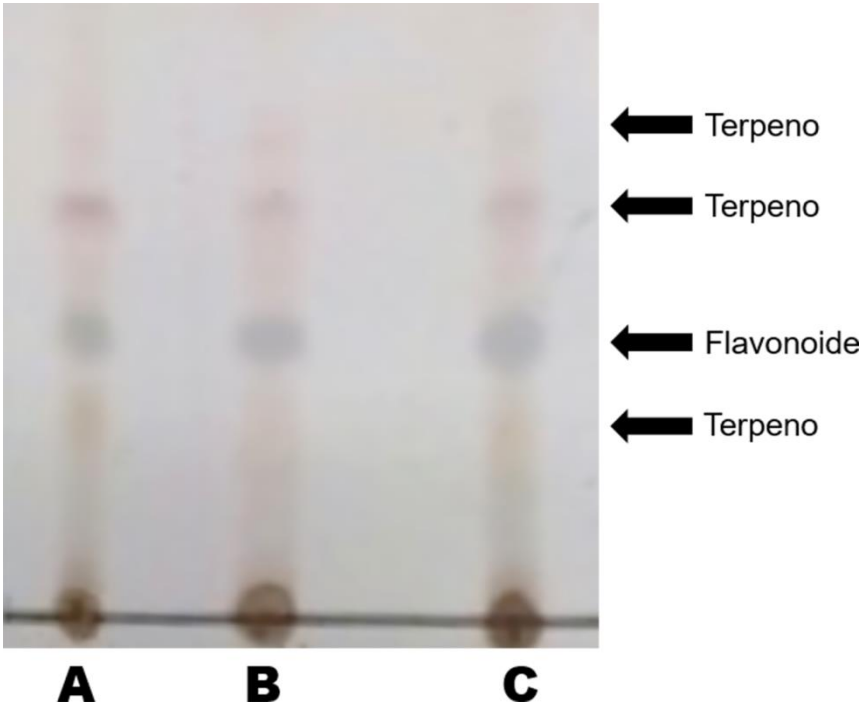
Against adult *R. microplus*, the Native genotype extract exhibited the best performance with 50% mortality at the 20% concentration, as well as a 51.3 % reduction in PRER. The Cunningham genotype produced mortality ≤20% and a PRER of 52.0 %, while the KX2 genotype also caused mortality ≤20% and had a PRER of 40.8 % (Table 1). No differences were observed between the genotypes in the chromatographic profile of the majority metabolites. Identified metabolites included terpenes and flavonoids, mainly of medium polarity (Figure 1).

Table 1: Mortality rate, reproductive efficiency index (REI) and percent reduction in estimated reproduction (PRER) in adult *Rhipicephalus microplus* ticks exposed to different concentrations of *Leucaena* spp. extracts

***Leucaena* spp.**	**Mortality (%)**	**REI (%)**	**PRER (%)**
Control	0	NA	NA
Native 20%	50	16.01	51.31
Native 10%	0	45.09	13.19
Cunningham 20%	10	4.04	52.04
Cunningham 10%	20	40.36	0.0
KX2 20%	20	42.05	44.55
KX2 10%	10	49.84	40.86

NA = not applicable.

Figure 1: Thin-layer chromatography of three *Leucaena* spp. genotypes: A) KX2; B) Native; and C) Cunningham. Hexane:AcOEt (8:2) system, oleum revealer



Discussion

The shrub legume *L. leucocephala* is distributed throughout the tropics and subtropics[21]. It is known to have defense mechanisms against fungi, bacteria and herbivorous insects[22,23], suggesting its use as an alternative in controlling the tick *R. microplus*.

Larval mortality rate was highest at the 50% concentration of the Cunningham (91.7 %) and KX2 (82.0 %) genotype extracts (Table 2). The lowest LC_{50} was 15.8 % for the 50% KX2 concentration, followed by 22.2 % for the Cunningham genotype. These results are similar to those reported in a study assessing the effect of an acetone *L. leucocephala* extract against different phases of *R. microplus*, with a resulting larval mortality rate of 66.79 %[12].

Table 2: Mortality rate and efficacy of *Rhipicephalus microplus* larvae exposed to different concentrations of ethanol extracts of three *Leucaena* spp. genotypes

***Leucaena* spp.**	**Concentration (%)**	**Mortality (%)**	**Efficacy (%)**
Control	NA	0	NA
Native	50.0	54.6	75.65
Native	40.0	49.5	67.3
Native	30.0	38.7	62.1
Native	20.0	28.0	57.0
Cunningham	50.0	91.7	92.7
Cunningham	40.0	78.4	82.0
Cunningham	30.0	62.4	71.0
Cunningham	20.0	29.5	59.3
KX2	50.0	82.0	90.7
KX2	40.0	77.7	83.0
KX2	30.0	64.9	82.0
KX2	20.0	58.4	72.7

NA = not applicable.

In adult ticks the LC_{50} for the Native genotype extract was attained with the 20% concentration, which also reduced PRER by 51.3 %. These results differ from a previous report in which no effect was observed against adult ticks[12]. Another study assessed the effect of defensive proteins and peroxidase produced by *L. leucocephala* when under stress versus adult *R. microplus* ticks, finding that, at a 0.1 mg/ml concentration, the defensive proteins reduced egg production by 8.5 %, larvae hatching by 47.7 % and larval efficacy by 56.3 %[13].

Other studies have evaluated the effect of extracts from other plants of the Fabaceae family against *R. microplus*. For example, when tested against adult *R. microplus*, a 20% concentration of extracts of leaves from *Habardia albicans* and *Cesalpinia gaumeri* produced low mortality rates (23 and 30 %, respectively), although the *H. albicans* extract did exhibit moderate inhibition of oviposition (54.4 %) and larvae hatching (48.7 %); in larvae, however, a 10% concentration caused 90 to 93 % mortality[7]. In an evaluation of an ethanol extract of *Dalbergia sissoo* against adult *R. microplus*, a 10% concentration was found to cause 85.0 % mortality and 55.9 % inhibition of oviposition, but had no effect on larvae hatching[24]. Also at a 10% concentration, ethanol extracts of the leaves of *Acacia farnesiana* and *A. harmandiana* were reported to produce low mortality rates in adult *R. microplus* (4.0 and 5.0 %, respectively)[25]. In another study, efficacy against *R. microplus* larvae was 82 % for a methanol extract of *Stylosanthes humilis* and 75 % for a methanol extract of *S. hamata* leaves[26].

The biological properties of *Leucaena* spp. genotypes can be attributed mainly to the presence and abundance of tannins, mimosine, phenols, coumarins and flavonoids, among others. A characterization of the composition of four *Leucaena* spp. genotypes (Cunningham, K636, native and KX2) found that all four contained different levels of condensed tannins and mimosine[27]. In the present study only terpenes and flavonoids were observed; together these constitute the majority metabolites in the evaluated extracts. Terpenes are known to cause mortality in *R. microplus*[28,29]. For instance, a study of essential oil from *Lippia sidoides* leaves found it to contain 67.6% thymol (a terpene compound), and that the oil caused over 95 % mortality in *R. microplus* larvae[30]. An evaluation of a natural extract of *Verbena officinalis* identified high flavonoids concentrations and found the extract to cause up to 67 % mortality *in vitro* against adult *R. microplus*[31]. In conjunction with the present results, these studies support the efficacy terpenes and flavonoids may have in controlling *R. microplus*.

Most of the natural compounds tested for tick control have modes of action that differ from commercial pharmaceutical treatments available for tick control, and may therefore be effective at controlling *R. microplus*. The present results suggest that ethanol extracts of *Leucaena* spp. are promising for control of *R. microplus* larvae but only moderately effective against adults. Studies have been done of the action mechanism of terpenes and flavonoids on arthropods. The monoterpenes contained in plant essential oils (D-limonene, myrcene, terpineol, linalool and pulegone) are known to be neurotoxic to the common fly (*Musca domestica*) and the German cockroach (*Blattella germanica*)[32]. In addition, thymol may strengthen the action of GABA (gamma-aminobutyric acid) receptors in undefined locations in insects[33]. Future research will need to focus on identifying the metabolites contained in ethanol extracts of *Leucaena* which have activity against *R. microplus*, be it individually or synergistically.

Conclusions and implications

The ethanol extracts of three *Leucaena* spp. genotypes were found to have good efficacy against *R. microplus* larvae and moderate efficacy against adults; they also reduce the percentage reduction in estimated reproduction. Flavonoids and terpenes were identified in the extracts and may be responsible for their acaricide activity. However, identification is still needed of the metabolites that produce this activity, be it individually or synergistically.

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